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Fluorimetry Study of *N*-(1-Pyrenyl)iodoacetamide-Labelled F-Actin

Local Structural Change of Actin Protomer both on Polymerization and on Binding of Heavy Meromyosin

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A fluorescent reagent, *N*-(1-pyrenyl)iodoacetamide, was conjugated to rabbit skeletal muscle actin at the site of the most reactive sulfhydryl group, and fluorescence characteristics (excitation and emission spectra, quantum yields, lifetimes) of the conjugate were investigated. Associated with polymerization of labelled G-actin, the fluorescence intensity at 407 nm, after excitation at 365 nm, was enhanced by a factor of about 25. It was reduced to about 25% on the binding of heavy meromyosin (or subfragment 1). The results suggest that binding of heavy meromyosin to the protomer of F-actin alters the local structure of the protomer towards a G-actin-like one.

Actin is found in a variety of cells and its interaction with various proteins and ions is essential in the activity of the cells. Actin assumes a double-stranded filament, F-actin, at high ionic strength (e.g. 0.1 M). F-actin binds 1 mol ADP and 1 mol divalent cation/mol actin protomer at the specific sites. In the presence of ATP F-actin undergoes a cyclic interaction with myosin, which results in an acceleration of the rate of hydrolysis of Mg-ATP by myosin. This interaction, in the muscle cell, generates the mechanical force for contraction. In the case of skeletal muscle, F-actin binds the group of regulatory proteins, tropomyosin–troponin, which control the actin–myosin interaction depending on the concentration of calcium ions in the cell.

It is believed that a conformational change of actin protomer occurs in association with these interactions, and many workers have studied how these possible changes occur and how they relate to the biological function of actin. So far various techniques have been developed and applied to monitor the conformational change of actin *in vitro* [1]. However, very cautious analysis is required for interpretation of the results obtained, since the signal which is considered to probe the conformational change of actin is often too small to allow a successful quantitative analysis. Moreover it is sometimes found that the signal change is perturbed by the effect of secondary phenomena, such as light scattering due to aggregation of proteins, which may not be related to the conformational change of actin. We, therefore, consider that it is important to search for probes which will reveal the anticipated conformational change of actin more sensitively. Recently we have found that a sulfhydryl reagent, *N*-(1-pyrenyl)iodoacetamide, labelling the fast reactive site of actin, exhibits a fluorescence spectrum which responds to the change in the state of actin more sensitively than those reported before; i.e. a 25-fold enhancement of fluorescence intensity associated with the polymerization of actin, and a reduction to 1/4 when heavy meromyosin was bound to F-actin in the absence of ATP. The result of our study will be given in this article.

EXPERIMENTAL PROCEDURE

Materials

Actin was extracted from acetone-treated powder of rabbit skeletal muscle into bidistilled water at 0°C. After clarification, the extract was made up to 0.5 M KCl (pH 7.6) [2] and polymerized actin was sedimented by centrifugation. The F-actin pellet obtained was washed with 0.1 M KCl and there dissolved in 0.2 mM ATP solution, containing 0.1 mM $CaCl_2$ 1 mM bicarbonate and 1 mM sodium azide, in order to give G-actin, which binds calcium ions at the specific site: G(Ca)-actin [3]. By addition of KCl (0.1 M) to G(Ca)-actin solution F(Ca)-actin was obtained. F(Ca)-actin was used throughout the present study. The protein concentration of actin was determined by the biuret reaction. The molecular weight of actin was taken to be 4.23×10^4 [4].

Heavy meromyosin was obtained by digestion of myosin with α-chymotrypsin (Sigma Chemical Co.) in the presence of 0.1 M NaCl and 1 mM $MgCl_2$ (pH 7.0) at 20°C for 10 min [5] Myosin subfragment 1 was obtained according to Weeds and Taylor [6]. The heavy meromyosin and subfragment 1 were purified by ammonium sulfate fractionation between 45% and 50% and between 55% and 67% respectively. The protein concentration was determined from ultraviolet absorbance using the absorption coefficient at 280 nm $A^{1\%}_{1\,\mathrm{cm}} = 6.45$ for heavy meromyosin and $A^{1\%}_{1\,\mathrm{cm}} = 7.5$ for subfragment 1 [5] The molecular weight was taken to be 3.4×10^5 for heavy meromyosin and 1.15×10^5 for subfragment 1.

N-(1-Pyrenyl)iodoacetamide was purchased from Molecular Probes (USA) and used without further purification Purity of the dye was checked by chromatography on a silica gel sheet.

Abbreviation. MalNEt, *N*-ethylmaleimide.

Methods

Steady-excitation fluorimetry was performed with a Hitachi MPF-2A spectrofluorimeter. Excitation and emission spectra were corrected as described previously [7]. Quantum yields were determined with quinine sulphonate in 0.05 M H_2SO_4 as a quantum yield of 0.50 [8]. Time-resolved fluorimetry was made with a single-photoelectron counting technique [7].

Absorption spectra were measured with Zeiss PMQ-II spectrophotometer.

Viscosity was measured with an Ostwald-type viscometer, with a flow time for the solvent of 107 s (20°C).

RESULTS

Conjugation of N-*(1-Pyrenyl)iodoacetamide to F-Actin*

In order to investigate the reactivity of sulfhydryl groups of actin towards *N*-(1-pyrenyl)iodoacetamide, F-actin (4.7 μM) was incubated with various concentrations of the dye in the presence of 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM ATP, 1 mM bicarbonate (pH 7.6) and 1 mM sodium azide. The reaction was continued in the dark at 20°C for 20 h, and then Whatman CF-11 cellulose was added to the solution at a final concentration of 1% (wt/wt). After removal of the cellulose adsorbing unreacted dye, the fluorescence intensity was measured at 386 nm after excitation at 347 nm. *N*-(1-Pyrenyl)iodoacetamide reacted preferentially with unique site of actin protomer, as is shown in Fig. 1. In order to determine this fast-reactive site we examined the reactivity of (*N*-(1-pyrenyl)iodoacetamide towards MalNEt-treated F-actin, which was prepared as follows. F-actin was reacted with MalNEt at 7°C for one day; the molar ratio of MalNEt to actin was 1.3. To this F-actin solution the dye was added under the same conditions as described above. It can be seen from Fig. 1 that the reaction of the dye towards the MalNEt-treated F-actin was completely inhibited. Since Elzinga and Collins [9] have shown that MalNEt reacts preferentially with Cys-373 of F-actin, it can be considered that the first mole of *N*-(1-pyrenyl)iodoacetamide will react with this residue.

On the basis of the results obtained above the labelled actin was prepared in the following way and used throughout the present study. The dye, dissolved in a mixture of 33% acetone and 67% dioxane, was gently added to the F-actin solution, in which the concentration of actin was 1.0 mg/ml and the solvent was the same as in the above experiment. The molar ratio of the dye to actin was 1 : 1. After removal of unreacted dye, the labelled F-actin was sedimented by centrifugation and resuspended in an aqueous solution containing 0.2 mM ATP, 0.1 mM $CaCl_2$, 2 mM imidazole-HCl (pH 7.0), 1 mM 2-mercaptoethanol and 1 mM sodium azide (buffer G). Actin, which was denatured during this procedure, was removed during a cycle of polymerization and depolymerization. Further purification of the labelled G-actin was made by gel chromatography on Sephacryl S-200 superfine (Pharmacia) by elution with buffer G. The degree of the labelling was found to be 70–95%. In this calculation the molar absorption coefficient of *N*-(1-pyrenyl)iodoacetamide-labelled G-actin, 2.2×10^4 M^{-1} cm^{-1} at 344 nm, was used, which was determined according to the method of Betcher-Lange and Lehrer [10].

Before doing a spectroscopic study the effect of the labelling on the polymerization of actin was investigated. The relationship between the concentration of actin and the specific viscosity in the solvent containing 0.1 M KCl, 1 m $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM phosphate buffer (pH 7. 0.2 mM ATP, 1 mM 2-mercaptoethanol and 1 mM sodi azide (buffer F) at 20°C is shown in Fig. 2B. It can be se that the critical concentration [11] of the labelled actin w about 0.005 mg/ml under these conditions, which is ess tially the same as that obtained for unlabelled actin [7]. I order to estimate the amount of denatured actin, measur ment were made on the amount of protein in the sup natant after centrifugation (4×10^4 rev./min for 90 min) the labelled F-actin (0.2 mg/ml) in buffer F. The protein co centration in the supernatant was estimated from the flu rescence intensity of the actin tryptophan. Only about of the total actin was found to have lost the ability polymerize.

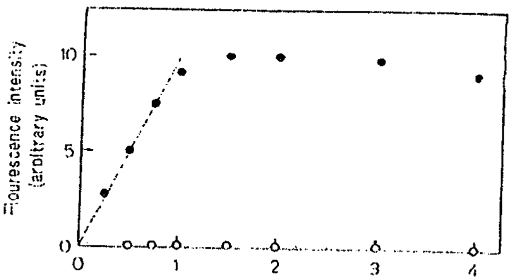

Fig. 1. *Fluorimetric titration of sulfhydryl groups of F-actin with N-* *pyrenyl)iodoacetamide.* Native (●) and MalNEt-treated F-actin (○) w reacted with various concentrations of the dye in the presence of 0.1 KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM ATP, 1 mM bicarbon and 1 mM sodium azide at 20°C for 20 h. After unreacted dye had be removed, the fluorescence intensity was measured at 386 nm af excitation at 347 nm (at 20°C)

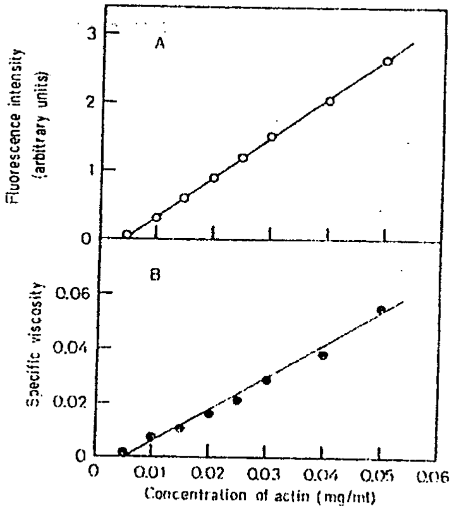

Fig. 2. *Static fluorescence intensity (A) and specific viscosity (B) of labelled actin as a function of the total concentration of actin.* The fl rescence intensity was measured at 407 nm after excitation at 365 n the slit width was 3 nm. Solvent conditions: 0.1 M KCl; 1 mM MgC 0.1 mM $CaCl_2$; 0.2 mM ATP; 10 mM phosphate buffer (pH 7.0); 1 m NaN_3; 1 mM 2-mercaptoethanol, at 20°C

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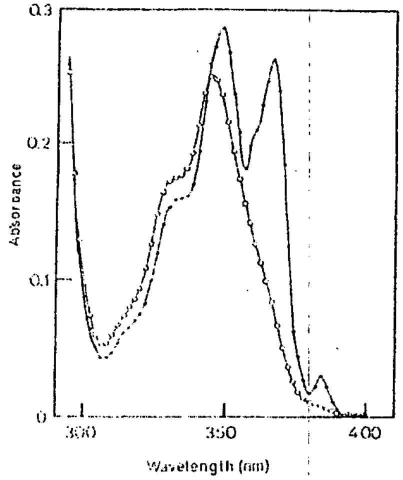

Fig. 3. *Absorption spectra of the labelled G (○) and F-actin (●) at 20°C.* Solvent conditions: 0.2 mM ATP, 0.1 mM CaCl₂, 2 mM imidazole-HCl (pH 7.0), 1 mM NaN₃ and 1 mM 2-mercaptoethanol for G-actin; in 0.1 M KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.2 mM ATP, 10 mM phosphate buffer (pH 7.0), 1 mM NaN₃ and 1 mM 2-mercaptoethanol for F-actin. The concentration of actin was 0.5 mg/ml

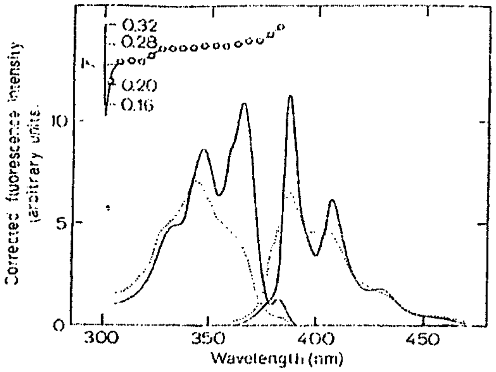

Fig. 4. *Fluorescence spectra (corrected) of the labelled G-actin (······) and F-actin (——).* The excitation spectra (left) were observed at 407 nm and the emission spectra (right) were obtained after excitation at 342 nm; the slit width was 3 nm. The excitation spectra are normalized so as to have the same intensity at 342 nm and the two emission spectra are normalized so as to obtain the same integrated intensity. The excitation polarization spectrum of the labelled F-actin is inserted: $\bar{r}$ is a time-averaged anisotropy. The slit width was 3 nm and emission was observed at 407 nm. The solvent conditions for G-actin and F-actin are the same as in Fig. 3

Fluorescence Spectra, Quantum Yields and Lifetimes of N-(1-Pyrenyl)iodoacetamide-Labelled Actin

Absorption and fluorescence spectra of the labelled G-actin and F-actin were investigated at 20°C. The concentration of the labelled actin was 0.2 mg/ml. At this concentration all of the labelled actin in buffer G exists as G-actin, while in buffer F the majority of actin (more than 97%) exists in the form of F-actin, as judged from viscosity measurements (Fig. 2). The absorption spectra of the labelled actin in buffers G and F (Fig. 3) showed that the polymerization of G-actin was accompanied by the appearance of peaks at 365 nm and 383 nm. The emission spectrum of the labelled F-actin showed a fine structure of vibration with peaks at 386 nm, 397 nm and 430 nm, while they were smeared in the spectrum of G-actin (Fig. 4).

Measurement of the fluorescence quantum yield of the labelled G and F-actin was made after excitation at 342 nm. The quantum yield obtained at 13°C was 0.083 for G-actin and 0.41 for F-actin. It should be noted that this increase in the quantum yield (approximately fivefold) on polymerization of G-actin corresponds to a 20–25-fold increase in the fluorescence intensity if observed at the emission peaks, i.e. at 386 nm or 407 nm, after excitation at 365 nm. Owing to this high sensitivity even a very small amount of F-actin can be easily distinguished from coexisting G-actin. Thus it can be seen from Fig. 2A that the critical concentration of the G–F equilibrium of actin can be determined more accurately by fluorimetric measurement than viscosity measurement. An important conclusion drawn from this result is that a large increase in the fluorescence intensity observed on polymerization is not due to an increase in the ionic strength of the solution but due to the association of actin protomers. This means that a change of the local structure near Cys-373 in G-actin occurs at the time of association of actin protomers. The structural change is not a prerequisite for polymerization.

The time-averaged anisotropy (inset of Fig 4) was almost independent of excitation wavelength in the region between 330 nm and 370 nm, suggesting that the two peaks of the excitation spectrum at 345 nm and at 365 nm belong to the same electronic excitation transition but have different vibrational modes; whereas the fluorescence quantum yield of the probe is largely dependent on excitation wavelength, as can be seen from comparison of the absorption and excitation spectra of both the labelled G and F-actin (Fig. 4 and 6). We give the following explanation of these results. The fluorophore has heterogeneous environments even in its ground state; for instance, some fraction of the fluorophore exists in a state with a very low quantum yield whose absorption spectrum exhibits an obscure structure similar to that of G-actin. The other fraction, however, exists in a different state with high quantum yield, whose absorption spectrum exhibits a fine structure of vibration as seen typically in the excitation spectrum obtained with the labelled F-actin. Thus the change in absorption spectrum on polymerization might be due to a change in the population of the two states. Such a possibility was also indicated in our previous study [7].

Neither the labelled G nor F-actin exhibited single-exponential fluorescence decay when excitation was performed at 358 nm and emission above 400 nm was recorded. Then the experimental fluorescence decay curves were analysed by using a method of least-squares, on the assumption that the fluorescence decay consists of a sum of the exponential functions [12]. The average fluorescence lifetime was calculated from the decay parameters obtained:

$$\langle\tau\rangle = \Sigma C_i \tau_i$$

where τ_i are the decay constants and C_i the relative amplitudes of the exponential decay components. The average fluorescence lifetime of the labelled G-actin was 3.99 ns, and 10.0 ns was obtained for the labelled F-actin (at 13°C). Apparently the ratio of the average lifetime of the labelled

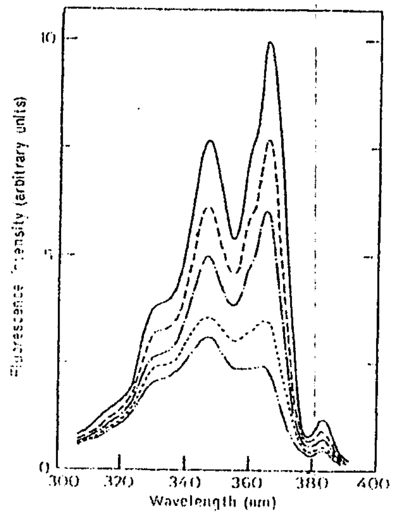

Fig. 5. *Excitation spectra of the labelled F-actin in the presence of various amounts of heavy meromyosin.* The molar ratio of heavy meromyosin to actin was 0 (·–), 0.125 (– –), 0.2 (· · ·), 0.5 (------), 2.2 (····). The slit width was 3 nm and emission was observed at 407 nm; an optical cut-off filter was set in the emission side for elimination of scattered light. The concentration of the labelled actin was 0.2 mg/ml and the degree of labelling of actin with the dye was 95%. Solvent conditions: 50 mM KCl; 2 mM $MgCl_2$; 0.1 mM $CaCl_2$; 10 mM phosphate buffer (pH 7.0); 1 mM NaN_3; 1 mM 2-mercaptoethanol, at 14 °C

G-actin to that of the labelled F-actin is not equal to the ratio of their quantum yields obtained above, because the excitation wavelength was different for these two measurements. Presumably the population of fluorophore in the high quantum yield would be smaller after excitation at 358 nm (lifetime measurement) than excitation at 365 nm (quantum yield measurement).

Absorption and Fluorescence Spectra of N-(1-Pyrenyl)maleimide-Labelled F-Actin in the Presence of Heavy Meromyosin or Subfragment 1

The labelled G-actin (2–4 mg/ml) was polymerized by addition of 0.1 M KCl, and ATP in the solution was removed by dialysis against the solution containing 0.1 M KCl, 1 mM $MgCl_2$ (or 0.05 M KCl, 2 mM $MgCl_2$), 10 mM phosphate buffer (pH 7.0), 1 mM 2-mercaptoethanol and 1 mM sodium azide. To this solution of the labelled F-actin, various amounts of heavy meromyosin or subfragment 1 were added; the final concentration of the labelled F-actin was 0.18–0.2 mg/ml.

Fig. 5 shows the excitation spectra of the labelled F-actin in the presence of various amounts of heavy meromyosin. With increasing concentration of heavy meromyosin added, the shape of the excitation spectrum changed: in particular, the peak at 365 nm was reduced more remarkably than the peak at 345 nm. On the other hand, the shape of the emission spectrum observed after excitation at 365 nm was almost independent of the amount of heavy meromyosin, though the fluorescence intensity integrated over emission was reduced. A striking change in the absorption spectrum of the labelled F-actin was observed on addition of heavy meromyosin. The open circles in Fig. 6 show the absorption spectrum obtained when an excess amount of heavy meromyosin (3.0 mg/ml) was added. Then the absorption peak at 365 nm disappeared, which was characteristic for the labelled F-actin (dashed li in Fig. 6). It should be noted that the absorption spectr obtained at complete saturation of heavy meromyosin bindi was almost indistinguishable from that of the labelled G-acti (continuous line in Fig. 6).

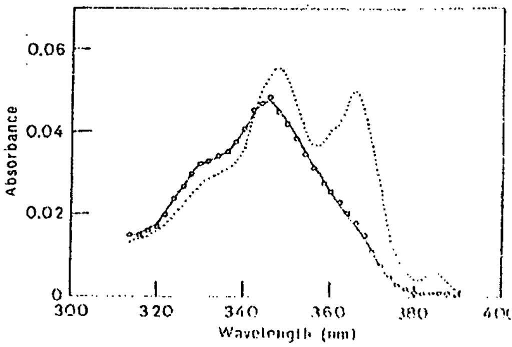

Fig. 6. *Absorption spectrum of the labelled F-actin in the presence of he meromyosin* (○). The molar ratio of heavy meromyosin to actin was 2 The concentration of the labelled actin was 0.2 mg/ml and the deg of labelling was 95%. A sample cuvette 0.5 cm in length was use Solvent conditions were the same as in Fig. 5. (····) Absorption spe trum of the labelled F-actin alone; (——) absorption spectrum of t labelled G-actin

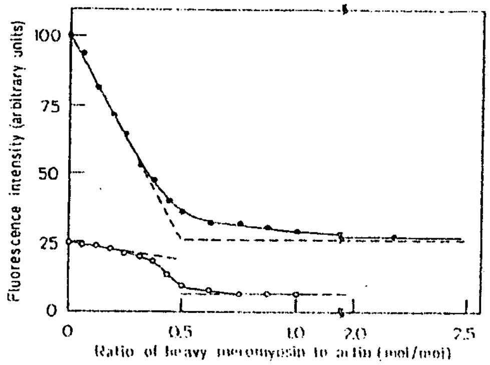

Fig. 7. *Fluorescence intensity as a function of the molar ratio of he meromyosin added to the labelled F-actin.* (●) Data obtained when t degree of labelling of actin was 95%. The concentration of F-actin w 0.2 mg/ml. (○) Data obtained when the labelled G-actin (95% labelle was diluted fourfold by co-polymerization with unlabelled G-actin. T fluorescence intensity was measured at 407 nm after excitation at 365 n Solvent conditions were the same as shown in Fig. 5

Fig. 7 shows the fluorescence intensity of the labelle F-actin as a function of the molar ratio of heavy meromyos to actin protomer (α). The change in the fluorescence intensi on increasing the amount of heavy meromyosin was found t depend on the labelling ratio of *N*-(1-pyrenyl)iodoacetami to actin (γ). The closed circles in Fig. 7 show the data ol tained at $\gamma = 0.95$. At this value of γ the fluorescence intensi decreased approximately linearly on increasing amount heavy meromyosin added. The extrapolation of the slo reached at $\alpha \approx 0.5$. The open circles in Fig. 7 show the da obtained at $\gamma = 0.24$, where the fluorescence intensity did n decrease linearly with increasing α; the initial decrease ($0 < \alpha < 0.3$) was less steep and a sharp decrease was found at close to 0.5. The initial slope of decrease in the fluoresce intensity increased monotonically as γ was increased (Table

Table 1. *Dependence of the initial slope (dΔ/dα) and F_∞/F_0 on the degree of labelling*

Here γ is the degree of labelling of actin with *N*-(1-pyrenyl)iodoacetamide; α is the molar ratio of heavy meromyosin to actin; Δ is the magnitude of decrease in the fluorescence intensity with reference to the maximum decrease, that is $\Delta = (F_0 - F)/(F_0 - F_\infty)$; F_0 and F_∞ are the fluorescence intensities observed at α = 0 and α = ∞ respectively. The fluorescence intensity was observed at 407 nm after excitation at 365 nm. The total concentration of actin was 0.18—0.20 mg/ml. Solvent conditions: 0.05 M KCl [or 0.1 M (a), or 0.03 M (b)]; 2 mM $MgCl_2$ [or 1 mM (a, b)]; 10 mM phosphate buffer (pH 7.0); 1 mM NaN_3; 1 mM 2-mercaptoethanol, at 14 °C [or 20 °C (a, b)]

γ	dΔ/dα	F_∞/F_0
0.95	2.0	0.27
0.75	1.7	0.25
0.46 (a)	0.76	0.26
0.46 (b)	0.85	0.24
0.24	0.48	0.26

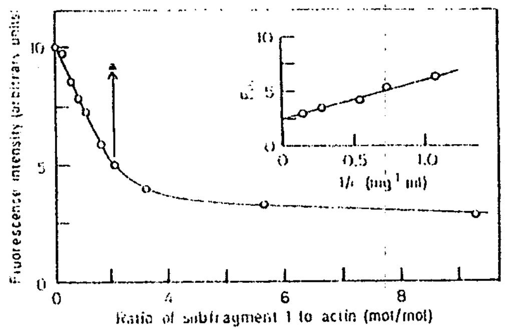

Fig. 8. *Fluorescence intensity as a function of the molar ratio of subfragment 1 added to the labelled F-actin.* c is the concentration of subfragment 1 added. Fluorescence intensity was measured at 407 nm after excitation at 365 nm. Solvent conditions: 0.1 M KCl; 1 mM $MgCl_2$; 10 mM phosphate buffer (pH 7.0); 1 mM 2-mercaptoethanol; 1 mM NaN_3 at 20 °C. The degree of labelling of F-actin (0.2 mg/ml) was 90%. (▲) Indicates the fluorescence intensity observed 1—3 min after addition of ATP (1 mM)

However, the saturation of decrease in the fluorescence intensity occurred at α ≈ 0.5 independently of γ. The ratio of the fluorescence intensity observed at α → ∞ (F_∞) to the intensity at α = 0 (F_0) was also independent of γ having a value around 0.25 (Table 1). These results can be simply explained by a lower binding affinity of heavy meromyosin to the labelled actin protomers than to unlabelled actin protomers in F-actin. However, since we measured only the fluorescence change of the labelled F-actin under the influence of heavy meromyosin and we do not know the actual amount of the bound heavy meromyosin, the binding constant was not determined.

Addition of subfragment 1 to the labelled F-actin brought about the similar changes in the absorption and fluorescence spectra as observed with heavy meromyosin. The fluorescence intensity at complete saturation of F-actin with subfragment 1 was 25% of that observed in the absence of subfragment 1 (Fig. 8). Immediately after ATP was added to the solution of the labelled actosubfragment 1 (1 mg/ml subfragment 1) at a final concentration of 1 mM, the fluorescence intensity recovered to about 90% of that observed in the absence of subfragment 1. It is, therefore, indicated that the change in the fluorescence intensity was reversible with respect to the binding of the myosin head to F-actin.

DISCUSSION

Previously Rich and Estes [13] found that F-actin at high ionic strength is resistant to proteolysis, and G-actin at low ionic strength is easily susceptible to proteolytic digestion by chymotrypsin and trypsin. They also found that G-actin (below the critical concentration) at high ionic strength is resistant to proteolysis. Thus they concluded that, by increase in ionic strength, a part of the G-actin polypeptide which is susceptible to proteolysis becomes resistant to proteolysis before polymerization. Actin in this state was named as 'F-monomer' by these authors. The present study showed that the local structure near Cys-373 of the 'F-monomer' is still in the same state as (or quite similar to) G-actin.

When heavy meromyosin was bound to *N*-(1-pyrenyl)-iodoacetamide-labelled F-actin, conspicuous changes in the absorption and fluorescence spectra were observed. The question is: what kind of change in the local environment of the probe can result in such a large change in the absorption spectrum as the observed on the binding of heavy meromyosin as well as on the G—F transformation? When the absorption spectra of *N*-(1-pyrenyl)iodoacetamide—2-mercaptoethanol conjugate were measured in various organic solvents with different polarities (i.e. hexane, dioxane, chloroform, ethanol) all of them were found to be relatively similar to the absorption spectrum of the labelled G-actin and none had a strong vibronic absorption band around 365 nm, as observed in the labelled F-actin (Fig. 3). It is, therefore, unlikely that the large change in the absorption spectrum of the labelled actin is solely due to a change in local polarity. Presumably a specific interaction between the fluorophore and a side-chain of F-actin gives rise to a strong vibronic band around 365 nm. The absorption spectrum at complete saturation of the labelled F-actin with heavy meromyosin was very similar to that of the labelled G-actin, while the excitation spectrum was still different from that of the labelled G-actin. On the other hand, the strong absorption band at 365 nm, which is characteristic of F-actin, almost disappeared on the binding of heavy meromyosin. It has been shown in the previous paper [7] that, under the influence heavy meromyosin binding, *N*-(1-pyrenyl)maleimide-labelled F-actin exhibits fluorescence decay, the decay parameters of which have values intermediate between those of G-actin and of F-actin alone. The present result has confirmed this point. Therefore, it is strongly suggested that binding of heavy meromyosin to F-actin alters the local conformation of each protomer, making it similar (but not identical) to that of G-actin. In the present study no essential difference between heavy meromyosin and subfragment 1 was observed in their effect on actin protomers.

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